



b

in CHO cells. (a) CHO cells transfected methionine, 100  $\mu$ Ci/ml. 1164 Ci/mmol as with anti-IFN- $\beta$  antibodies, and immunok culture represents data from wild-type copies of pM17 were superinduced and onine as indicated; the supernatants were analyzed on SDS-PAGE.

side, 10% ethylene glycol in loading buffer) is added to the column; after 15 min on the column, material is collected, dialyzed against PBS, and titrated for IFN activity.

#### Concluding Comments

We have applied the techniques described above to establish CHO cell lines that can be induced to secrete glycosylated and unglycosylated forms of human IFN- $\beta$ . While initial levels can be low, about 100 units/nl, we have been able to coamplify the IFN- $\beta$  gene along with the DHFR gene by methotrexate selection and to obtain very high levels of IFN expression in selected cell lines.<sup>5</sup> Immunoprecipitates of [<sup>35</sup>S]methionine-labeled supernatants from initial transfectants of CHO cells expressing wild-type IFN- $\beta$  (pM17) or a mutant IFN- $\beta$  (pMB-1) gene where glutamine was substituted for asparagine at position 80 are shown in Fig. 1a. Cells expressing the modified IFN- $\beta$  (pMB-1) secrete a form of IFN- $\beta$  that migrates at 18,500 D on SDS-PAGE, and do not produce the major 23,000 form seen in supernatants from cells expressing unmodified IFN- $\beta$  (pM17). An 18,500 D form of IFN- $\beta$  is also produced from the wild-type gene and appears to be unglycosylated, as shown in Fig. 1b. The 18,500 D form of IFN- $\beta$  produced from the pMB-1 gene appears to be unglycosylated confirming that the asparagine residue at position 80 is the site for glycosylation in native IFN- $\beta$ . Preliminary results indicate that the unglycosylated IFN- $\beta$  produced and secreted from the mutant gene has a much lower specific biological activity than glycosylated IFN- $\beta$ . The availability of these cell lines will permit us to evaluate further the role of glycosylation in the activity and physical properties of IFN- $\beta$ .

#### [58] Procedures for *in Vitro* DNA Mutagenesis of Human Leukocyte Interferon Sequences

By THOMAS M. DECHIARA, FRAN ERLITZ, and S. JOSEPH TARNOWSKI

#### Introduction

The mutagenesis of cloned genes has become a powerful research tool in the analysis of protein function (for a review, see Dalbadie-McFarland and Richards<sup>1</sup>). This chapter describes the production of recombinant

<sup>1</sup> G. Dalbadie-McFarland and J. H. Richards, *Annu. Rep. Med. Chem.* 18, 237 (1983).

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human leukocyte interferon A (IFN- $\alpha$ A) analogs to understand better how the structure of IFN- $\alpha$ A is related to its biological activity. Two approaches were taken to specifically mutate the IFN- $\alpha$ A gene. In one approach, synthetic deoxyoligonucleotides were employed to replace a desired region of IFN- $\alpha$ A DNA located between convenient restriction enzyme recognition sites. These deoxyoligonucleotides were identical in sequence to the segment they replaced, except for a single codon change. The mutated genes encoded the substitution of cysteine residues 1 and 98 in the protein with glycine and serine, respectively. In the second approach, site-specific mutations were directed by single deoxyoligonucleotides in a procedure which utilized a heteroduplex of plasmid DNA. These mutations were in the IFN- $\alpha$ A gene region which encoded the carboxy terminal 27 amino acids.

#### Solution and Materials

TAE: 40 mM Tris-acetate, pH 7.8, 5 mM NaOAc, 2 mM EDTA

TE: 10 mM Tris · HCl, pH 7.4, 1 mM EDTA

Ligation solution: 60 mM Tris · HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 400  $\mu$ M ATP. All ligation reactions were performed at 15° for 12 hr in a volume of 10  $\mu$ l except where indicated.

Restriction endonucleases: *Bst*II, *Eco*RI, *Hinf*I, *Pvu*II, and *Sau*3A1 were from New England Biolabs. The buffer conditions used were described by the manufacturer.

Plasmids: (1) pRC23 is described in detail in this volume.<sup>2</sup> (2) pRC234 is a derivative of pRC23 in which the *Pvu*II restriction endonuclease site was deleted by a brief *Bal*31 exonuclease digestion followed by ligation of the blunt ends to recircularize the plasmid. The IFN- $\alpha$ A gene was inserted into the unique *Eco*RI site of these vectors.

Klenow fragment of *E. coli* DNA Polymerase I and T<sub>4</sub>-DNA ligase from Boehringer Mannheim.

#### Substitution of Cys 1 and Cys 98 Residues

The nucleotide sequence of the IFN- $\alpha$ A DNA<sup>3</sup> predicts cysteine residues at positions 1, 29, 98, and 138 in the protein (Fig. 1a). Disulfide bond assignments,<sup>4</sup> together with selective reduction studies,<sup>5</sup> have suggested

<sup>2</sup> R. Crowl, this volume [55].

<sup>3</sup> S. Pestka, *Arch. Biochem. Biophys.* 221, 1 (1983).

<sup>4</sup> R. Wetzel, *Nature (London)* 289, 606 (1981).

<sup>5</sup> R. Wetzel, H. L. Levine, D. A. Estell, and S. Shire, *J. Cell. Biochem., Suppl.* 6, 89 (1982).

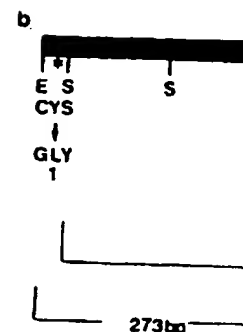
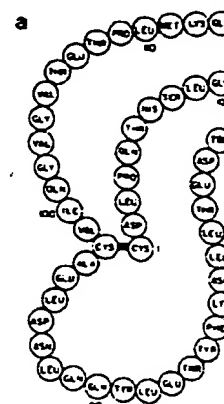


FIG. 1. (a) Schematic representation of the IFN- $\alpha$ A gene. The gene encoding IFN- $\alpha$ A is carried on a plasmid of about 5000 bp of coding sequences. (b) Schematic representation of the IFN- $\alpha$ A gene. The gene encoding IFN- $\alpha$ A is carried on a plasmid of about 5000 bp of coding sequences. E, *Eco*RI; S, *Sau*3A1; P, *Promoter*.

that, in contrast to the Cys 98 bond is less stable and is not as measured *in vitro*. The 98 residues, or both, by close proximity to the biological activity of IFN- $\alpha$ A (Table I) were used to replace the Cys 98 residue between the *Eco*RI and *Promoter* sites, or between the *Promoter* and Cys 1, or between the *Promoter* and Cys 98.

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its biological activity. Two  
the IFN-αA gene. In one  
were employed to replace a  
between convenient restriction  
nucleotides were identical in  
ept for a single codon change.  
of cysteine residues 1 and 98  
spectively. In the second ap-  
ted by single deoxyoligonu-  
teroduplex of plasmid DNA.  
ie region which encoded the

nM NaOAc, 2 mM EDTA  
EDTA  
H 7.5, 10 mM MgCl<sub>2</sub>, 10 mM  
All ligation reactions were per-  
10 μl except where indicated.  
RI, *Hinf*I, *Pvu*II, and *Sau*3AI  
e buffer conditions used were

ail in this volume.<sup>2</sup> (2) pRC234  
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/31 exonuclease digestion fol-  
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he unique *Eco*RI site of these

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*J. Cell. Biochem., Suppl.* 6, 89 (1982).

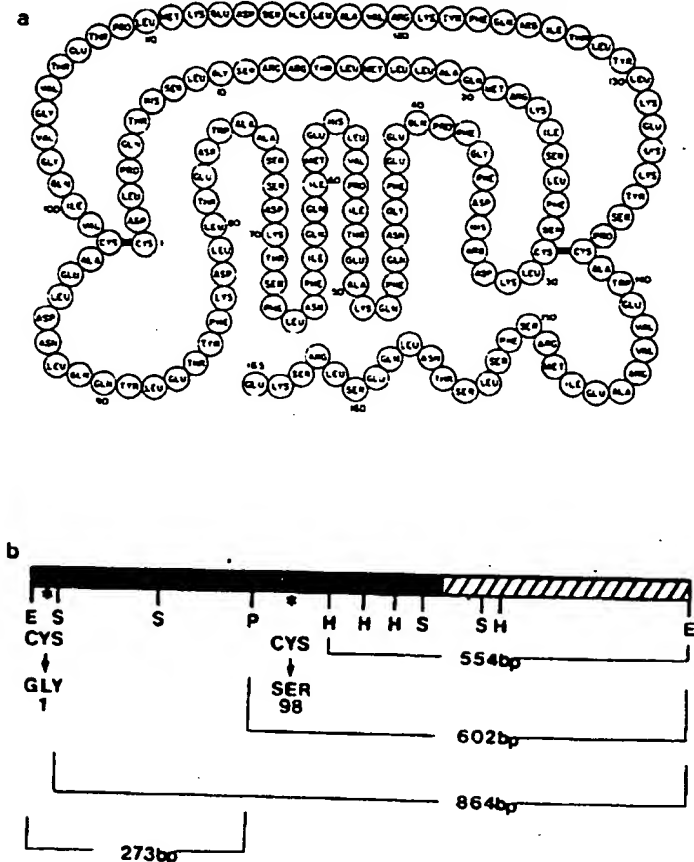


FIG. 1. (a) Schematic representation of IFN-αA (taken with permission from Pestka<sup>3</sup>). (b) The gene encoding IFN-αA is carried on an *Eco*RI fragment.<sup>6,7</sup> The solid region represents about 500 bp of coding sequences and the hatched region, about 350 bp of 3' untranslated DNA. E, *Eco*RI; S, *Sau*3AI; P, *Pvu*II; H, *Hinf*I.

that, in contrast to the Cys 29/Cys 138 disulfide bond, the Cys 1/Cys 98 bond is less stable and is not a requisite for the antiviral activity of IFN-αA as measured *in vitro*. Therefore, substitution of either the Cys 1 or Cys 98 residues, or both, by closely related amino acids should have no effect on the biological activity of the protein. Synthetic deoxyoligonucleotides (Table I) were used to replace the amino terminal coding region of the gene between the *Eco*RI and *Sau*3AI restriction sites for the substitution of Cys 1, or between the *Pvu*II and *Hinf*I sites to substitute for Cys 98

TABLE I  
SYNTHETIC DNA TO REPLACE PARENTAL IFN- $\alpha$ A GENE FRAGMENTS\*

Synthetic deoxyoligonucleotide	Sequence
1A (11-mer)	<div style="text-align: center;">             I Gly              AATTCATGGGC           </div>
1B (11-mer)	<div style="text-align: center;">             EcoRI             <div style="display: inline-block; vertical-align: middle;">               AATTCATGGGC GTACCCGCTAG             </div>             Sau3AI           </div>
98A (18-mer)	<div style="text-align: center;">             PvuII              CTGAATGACCTGGAAGCC           </div>
98B (27-mer)	<div style="text-align: center;">             PvuII              GACTTACTGGACCTTCGGTCGCACTAT           </div>
98C (28-mer)	<div style="text-align: center;">             98 Ser              AGCGTGATACAGGGGGTGGGGGTGACAG           </div>
98D (22-mer)	<div style="text-align: center;">             98 Ser              AGCGTGATACAGGGGGTGGGGGTGACAG              GTCCCCCACCCTGTCTGA           </div>

\* Dashed line, restriction site overhang or blunt end; solid line, 9 base complementary sequence for the ligation of 98A, B, C, and D.

(Fig. 1b).<sup>6,7</sup> Each set of synthetic DNA molecules encoded one codon change. The TGT codon for Cys in the parental gene was replaced by GGC (Gly) or by AGC (Ser) where indicated (Table I).

Three mutated IFN- $\alpha$ A genes were constructed: one encoding the Gly 1 substitution, one encoding Ser 98, and one encoding both. The incorporation of glycine in the first amino acid position was achieved by the ligation of a 50-fold molar excess of synthetic deoxyoligonucleotides 1A and 1B (Table I) to the 864 bp *Sau3AI*/*EcoRI* gene fragment. This fragment was generated by *Sau3AI* partial digestion of IFN- $\alpha$ A DNA, necessitated by multiple *Sau3AI* restriction sites (Fig. 1b). Mutagenesis for the incorporation of Ser 98 resulted from the ligation of a 50-fold molar excess of deoxyoligonucleotides 98A, 98B, 98C, and 98D (Table I) to both the 273 bp *EcoRI*/*PvuII* fragment and the 554 bp *HinfI*/*EcoRI* fragment. The larger fragment was generated by *HinfI* partial digestion of the 602 bp *PvuII*/*EcoRI* gene fragment (Fig. 1b). The double substitution of Gly 1

<sup>6</sup> S. Maeda, R. McCandliss, M. Gross, A. Sloma, P. C. Familletti, J. M. Tabor, M. Evinger, W. P. Levy, and S. Pestka, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7010 (1980).

<sup>7</sup> D. V. Goeddel, E. Yelverton, A. Ullrich, H. L. Heyneker, G. Miozzari, W. Holmes, P. H. Seeburg, T. Dull, L. May, N. Stebbing, R. Crea, S. Maeda, R. McCandliss, A. Sloma, J. M. Tabor, M. Gross, P. C. Familletti, and S. Pestka, *Nature (London)* **287**, 411 (1980).

and Ser 98 was encoded by 1 (Gly 1) and the 602 bp *PvuII*.

Following ligation, the D) tated genes were inserted into *E. coli* as described in this v fermenters, harvested, and th homogenizer.<sup>8</sup> Each crude ex lized anti-leukocyte interferoi washed to remove extraneous were desorbed with a dilute s analogs were analyzed by SD mercaptoethanol, and assaye from a challenge by vesicular

Each of the three analogs p mg protein, identical to that c mation of the Cys 1/Cys 98 dis activity of the protein. In addi monoclonal antibody LI-8 ind not involved in the domain re

Analysis of the purified a cates the sulfhydryl groups at r lecular disulfide bond format substituted analog migrated al with some dimer form observ pattern of parental IFN- $\alpha$ A (li analog possessed a much high that the Cys 1 sulfhydryl group ular disulfide bond formation t forms were disulfide bonded w 1/Cys 1 dimer to "slow" mon ethanol (lane D). The Gly 1/Ser a "slow" monomer form only ( E) appeared to be susceptible t ance of a discrete fragment w appearance of this fragment w traction procedure.

<sup>8</sup> S. J. Tarnowski and R. A. Liptak, A  
<sup>9</sup> T. Staehelin, D. S. Hobbs, H. Kung, (1981).

<sup>10</sup> U. K. Laemmli, *Nature (London)* **22**  
<sup>11</sup> P. C. Familletti, S. Rubinstein, and S

αA GENE FRAGMENTS

quence

San3A1  
AGCC  
TCGGTCGCACTAT

GGTGGGGGTGACAG  
CCACCCCCACTGTCTCA

J: solid line, 9 base complementary

molecules encoded one codon  
parental gene was replaced by  
ed (Table I).  
structed: one encoding the Gly  
e encoding both. The incorpo-  
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(Fig. 1b). Mutagenesis for the  
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and 98D (Table I) to both the  
p *HinfI*/*EcoRI* fragment. The  
partial digestion of the 602 bp  
double substitution of Gly 1

7. Familletti, J. M. Tabor, M. Evinger,  
U.S.A. 77, 7010 (1980).  
Heyneker, G. Miozzari, W. Holmes,  
Crea, S. Maeda, R. McCandliss, A.  
S. Pestka, *Nature (London)* 287, 411

and Ser 98 was encoded by ligation of the 273 bp *EcoRI*/*PvuII* fragment (Gly 1) and the 602 bp *PvuII*/*EcoRI* fragment (Ser 98).

Following ligation, the DNAs were digested with *EcoRI* and the mutated genes were inserted into the *EcoRI* site of pRC23 for expression in *E. coli* as described in this volume.<sup>2</sup> *E. coli* cells were grown in 10-liter fermenters, harvested, and the cell pastes were lysed in a Manton-Gaulin homogenizer.<sup>8</sup> Each crude extract was passed over a column of immobilized anti-leukocyte interferon monoclonal antibody.<sup>9</sup> The columns were washed to remove extraneous *E. coli* proteins and the analog proteins were desorbed with a dilute acetic acid solution. Samples of the purified analogs were analyzed by SDS-PAGE<sup>10</sup> in the presence or absence of 2-mercaptoethanol, and assayed for their ability to protect MDBK cells from a challenge by vesicular stomatitis virus *in vitro*.<sup>11</sup>

Each of the three analogs possessed a specific activity of 2 × 10<sup>8</sup> units/mg protein, identical to that of parental IFN-αA. This confirms that formation of the Cys 1/Cys 98 disulfide bond is not necessary for the antiviral activity of the protein. In addition, the protein analogs were purified with monoclonal antibody LI-8 indicating that Cys 1 or Cys 98 residues were not involved in the domain recognized by this immunoglobulin.

Analysis of the purified analogs by nonreducing SDS-PAGE implicates the sulfhydryl groups at positions 1 and 98 to be involved in intermolecular disulfide bond formation. As illustrated in Fig. 2, the Gly 1-substituted analog migrated almost entirely as a "slow" monomer form with some dimer form observed (lane B), compared with the migration pattern of parental IFN-αA (lane A). In contrast, the Ser 98-substituted analog possessed a much higher dimer content (lane C). This indicates that the Cys 1 sulfhydryl group is a more active participant in intermolecular disulfide bond formation than the Cys 98 sulfhydryl. That the dimer forms were disulfide bonded was demonstrated by conversion of the Cys 1/Cys 1 dimer to "slow" monomer form in the presence of 2-mercaptoethanol (lane D). The Gly 1/Ser 98 double-substituted analog migrated as a "slow" monomer form only (lane E). The "slow" monomer form (lane E) appeared to be susceptible to proteolysis as evidenced by the appearance of a discrete fragment which migrated at about 14,000 Da. The appearance of this fragment was dependent on subtle changes in the extraction procedure.

<sup>8</sup> S. J. Tarnowski and R. A. Liptak, *Adv. Biotechnol. Processes* 2, 271 (1983).  
<sup>9</sup> T. Staehelin, D. S. Hobbs, H. Kung, C. Y. Lai, and S. Pestka, *J. Biol. Chem.* 256, 9750 (1981).  
<sup>10</sup> U. K. Laemmli, *Nature (London)* 227, 680 (1970).  
<sup>11</sup> P. C. Familletti, S. Rubinstein, and S. Pestka, this series, Vol. 78, p. 387.

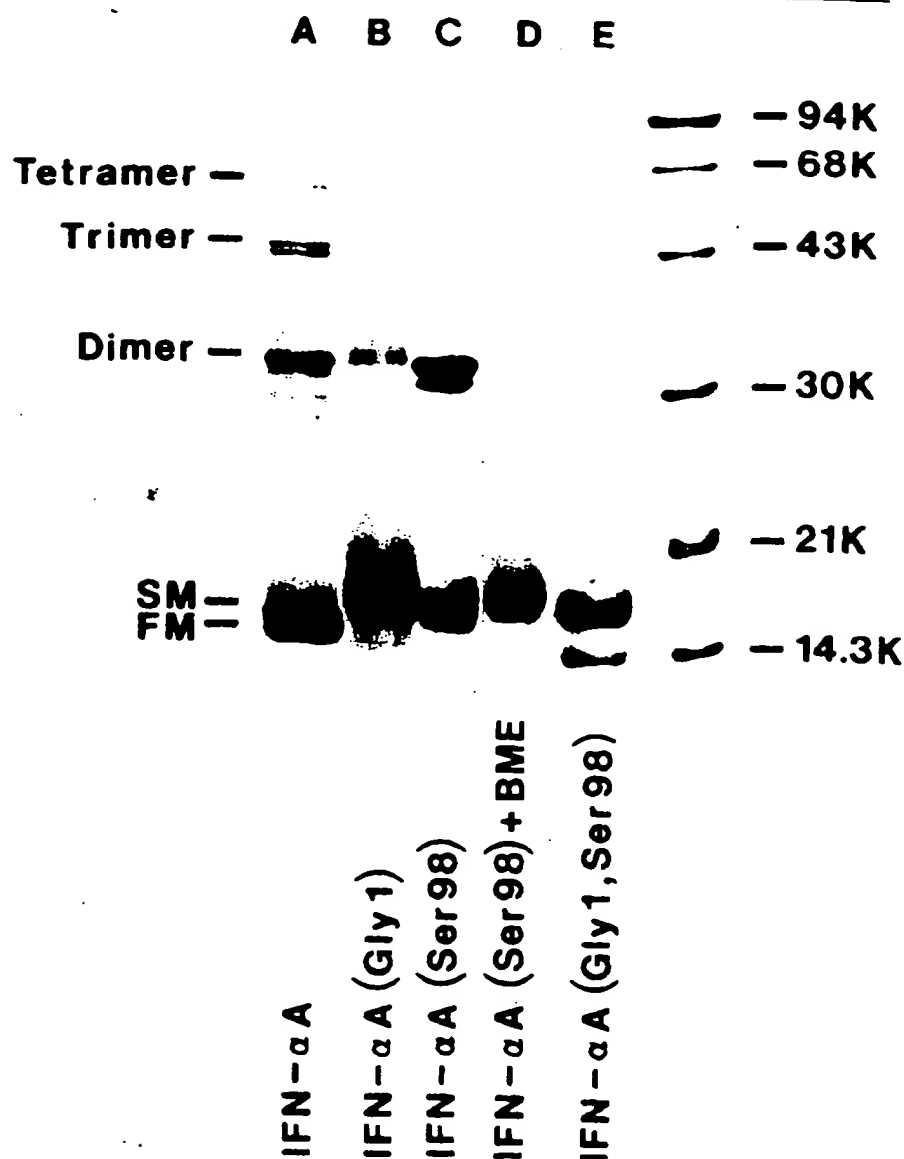


FIG. 2. Coomassie Brilliant blue-stained SDS-PAGE analysis of Cys 1/Cys 98 substituted IFN- $\alpha$ A analogs. Cells produced parental IFN- $\alpha$ A (*E. coli* W 3110 *trp* R-lac<sup>2</sup>/pLiFA *trp* 55), under *trp* promoter-operator control mechanisms.<sup>7</sup> IFN- $\alpha$ A (Gly 1), IFN- $\alpha$ A (Ser 98), or IFN- $\alpha$ A (Gly 1, Ser 98) (*E. coli* RR1/pRK248clts) were produced at 42° as described by R. Crowl.<sup>2</sup> Frozen cell pastes were homogenized to crude cell extracts<sup>8</sup> and interferon

### Modification of the IFN- $\alpha$ A C

Mutagenesis of the IFN- $\alpha$ A C-terminal 27 amino acids (139–165) DNA heteroduplex<sup>13</sup> (Fig. 3). the mutagenesis can be performed that mutations can be screened with the M13 system<sup>14</sup>; (2) the requirement of exonuclease II from a nick introduced by a amount of ethidium bromide<sup>15</sup>; purified from the linearized at 15% of the resulting transformation is a deletion (as was the case below), or a base change to intact mutants can easily be screened by

### Formation and Isolation of He

Use of the three restriction enzymes of opened plasmids: a linear fragment with a 550 bp *Pvu*II/*Bst*EII fragment. be no larger than about 12% of the total. make it more difficult to resolve linearized molecules. To obtain

<sup>12</sup> M. M. Bradford, *Anal. Biochem.* 72,

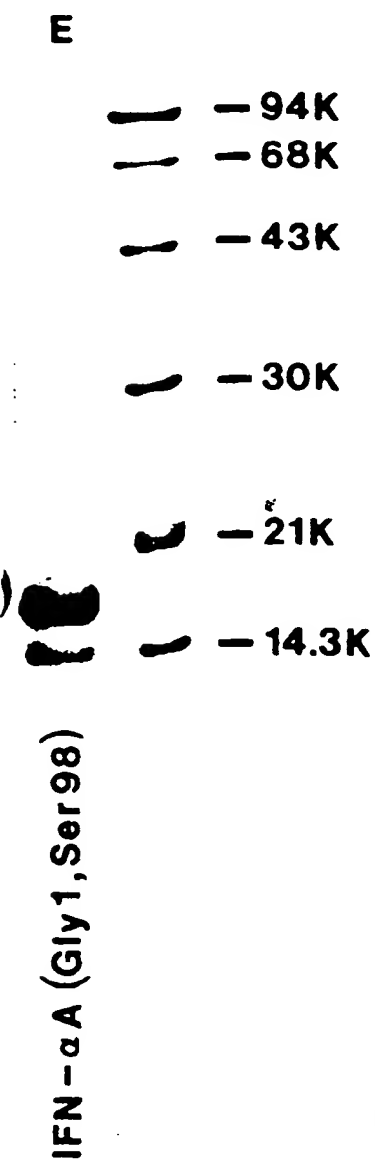
<sup>13</sup> A. Oka, K. Sugimoto, H. Sasaki, and

<sup>14</sup> G. Winter, A. K. Fersht, A. J. Wilkins 756 (1982).

<sup>15</sup> G. Dalbadie-McFarland, L. W. Cohen Richards, *Proc. Natl. Acad. Sci. U.S.A.*

<sup>16</sup> H. C. Birnboim and J. Doly. *Nucleic*

was purified on an immunosorbent column following protein determination<sup>12</sup> aliquots of analyzed by electrophoresis<sup>10</sup> on a 12.5% Electrophoresis of the samples under non-reducing buffer without 2-mercaptoethanol. Lane C. 10  $\mu$ g IFN- $\alpha$ A (Gly 1), nonreduced; lane E. 10  $\mu$ g IFN- $\alpha$ A (Ser 98), reduced; lane E. 10  $\mu$ g protein markers from Bio-Rad Laboratories: 68,000, bovine serum albumin; 43,000, ovalbumin; 30,000, soybean trypsin inhibitor; 14,300, lysozyme. monomer with both disulfide bonds intact



AGE analysis of Cys 1/Cys 98 substi-  
- $\alpha$ A (*E. coli* W 3110 *trp* R-lac<sup>2</sup>/pLiFA  
isms.<sup>7</sup> IFN- $\alpha$ A (Gly 1), IFN- $\alpha$ A (Ser  
:Its) were produced at 42° as described  
to crude cell extracts<sup>4</sup> and interferon

Modification of the IFN- $\alpha$ A Carboxy-Terminus

Mutagenesis of the IFN- $\alpha$ A gene region encoding the carboxy terminal 27 amino acids (139–165) was performed by generating a plasmid DNA heteroduplex<sup>13</sup> (Fig. 3). The advantages of this method are that (1) the mutagenesis can be performed directly on the expression plasmid so that mutations can be screened rapidly without subcloning as is necessary with the M13 system<sup>14</sup>; (2) the formation of heteroduplexes eliminates the requirement of exonuclease III digestion to create a single-stranded gap from a nick introduced by a restriction endonuclease and a calibrated amount of ethidium bromide<sup>15</sup>; and (3) since heteroduplexes can easily be purified from the linearized and gapped homoduplexes, between 5 and 15% of the resulting transformants contain mutant plasmids. If the mutation is a deletion (as was the case for IFN- $\alpha$ A gene mutations described below), or a base change to introduce a restriction site, then the transformants can easily be screened by a rapid plasmid isolation protocol.<sup>16</sup>

Formation and Isolation of Heteroduplex

Use of the three restriction enzymes (Fig. 3) generated two populations of opened plasmids: a linearized plasmid, and a linearized plasmid with a 550 bp *PvuII/BstEII* fragment removed. It is desirable that the gap be no larger than about 12% of the plasmid's size. Excessively larger gaps make it more difficult to resolve the heteroduplexes from homoduplex linearized molecules. To obtain sufficient quantities of heteroduplex, at

<sup>12</sup> M. M. Bradford, *Anal. Biochem.* 72, 248 (1976).  
<sup>13</sup> A. Oka, K. Sugimoto, H. Sasaki, and M. Takanami, *Gene* 19, 59 (1982).  
<sup>14</sup> G. Winter, A. K. Fersht, A. J. Wilkinson, M. Zoller, and M. Smith, *Nature (London)* 299, 756 (1982).  
<sup>15</sup> G. Dalbadie-McFarland, L. W. Cohen, A. D. Riggs, C. Morin, K. Itakura, and J. H. Richards, *Proc. Natl. Acad. Sci. U.S.A.* 79, 6409 (1982).  
<sup>16</sup> H. C. Birnboim and J. Doly, *Nucleic Acids Res.* 7, 1513 (1979).

was purified on an immunosorbent column of immobilized monoclonal antibody LI-8.\* Following protein determination<sup>12</sup> aliquots of each antibody pool were dried by evaporation and analyzed by electrophoresis<sup>10</sup> on a 12.5% polyacrylamide slab gel containing 0.1% SDS. Electrophoresis of the samples under nonreducing conditions was performed with sample buffer without 2-mercaptoethanol. Lane A, 15  $\mu$ g parental IFN- $\alpha$ A, nonreduced; lane B, 10  $\mu$ g IFN- $\alpha$ A (Gly 1), nonreduced; lane C, 10  $\mu$ g IFN- $\alpha$ A (Ser 98), nonreduced; lane D, 10  $\mu$ g IFN- $\alpha$ A (Ser 98), reduced; lane E, 10  $\mu$ g IFN- $\alpha$ A (Gly 1, Ser 98), nonreduced. Standard protein makers from Bio-Rad Laboratories (Rockville, NY): 94,000, phosphorylase b; 68,000, bovine serum albumin; 43,000, ovalbumin; 30,000, carbonic anhydrase; 21,000, soybean trypsin inhibitor; 14,300, lysozyme, all reduced. SM, "slow" monomer; FM, "fast" monomer with both disulfide bonds intact.

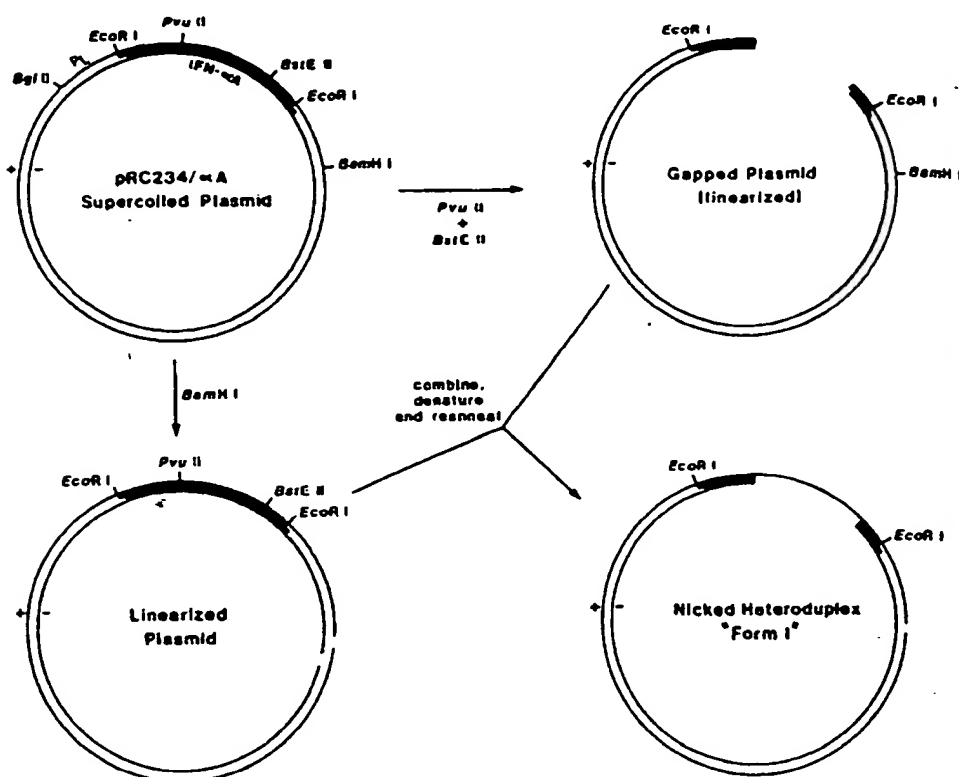


FIG. 3. Formation of plasmid DNA heteroduplex as a base for site-directed mutagenesis. See text for details. pRC 234 is a derivative of pRC 23 with the *PvuII* site in the plasmid deleted.

least 1  $\mu\text{g}$  of supercoiled plasmid was used for each of the two restriction enzyme digests. After digestion, the linearized plasmids were purified by electrophoresis through agarose, recovered, and precipitated with ethanol.

Purified linearized plasmids were resuspended in 25  $\mu\text{l}$  of  $\text{H}_2\text{O}$  and combined in an Eppendorf tube. After addition of 50  $\mu\text{l}$  of a solution containing 0.2 *N* NaOH and 40 mM EDTA, the plasmids were denatured at 23°. After 10 min, 10  $\mu\text{l}$  of 2 *M* Tris (1.8 *M* Tris  $\cdot$  HCl, 0.2 *M* Tris base) and 110  $\mu\text{l}$  of deionized formamide were added. Annealing of single-stranded DNA proceeded for about 3 hr at 23°. Two heteroduplex forms resulted during the annealing process, Form I from the annealing of the "+" strand of the linearized plasmid with the "-" strand of the gapped

plasmid, and Form II from single-stranded gap of 550 bp. desired mutations on the "+" necessary to separate the two mononucleotides were complementary.

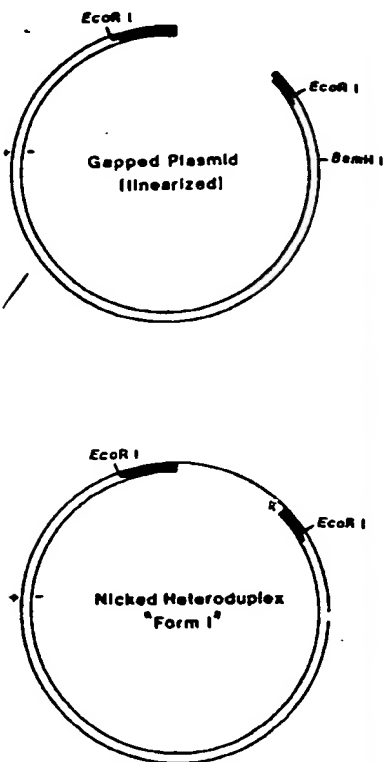
After annealing, 300  $\mu\text{l}$  of were added and the heteroduplex with ethanol. After the second cold ethanol and dried under vacuum by electrophoresis through forms migrated with nicked plasmid forms.

To recover the heteroduplex. The gel strip containing dialysis bag with 1 ml of 1% bromide (50  $\mu\text{g}/\text{ml}$ ). The heteroduplex long wavelength UV as they were. After electrophoresis, the heteroduplex with four 1 ml rinses of the mM Tris  $\cdot$  HCl, pH 7.4, 1 *M* used to reduce the volume of added and the heteroduplex extracted with chloroform/isoamyl alcohol (1:1), dried, and resuspended in water (as measured by visual comparison on agarose gel) was recovered from the necessary due to the strong association of the heteroduplex and agarose heteroduplex through nitrocellulose high affinity for the filter.

#### Introduction of Deletions in

Deletions in plasmid DNA were introduced by proteins which were terminally labeled (Fig. 1a). The three deoxyoligonucleotides loop-out of sequences in the immediately downstream of the Arg-149. By designing the synthesis in the "+" strand on the 5' side of the 3' side, the intervening sequence mononucleotide will loop-out as





a base for site-directed mutagenesis. with the *PvuII* site in the plasmid

or each of the two restriction  
ized plasmids were purified  
ered, and precipitated with  
  
pended in 25  $\mu$ l of H<sub>2</sub>O and  
dition of 50  $\mu$ l of a solution  
the plasmids were denatured  
f Tris · HCl, 0.2 M Tris base)  
added. Annealling of single-  
23°. Two heteroduplex forms  
n I from the annealing of the  
he “-” strand of the gapped

plasmid, and Form II from annealing of the other strands. In Form I, a single-stranded gap of 550 nucleotides was created in the region of the desired mutations on the “+” strand in the IFN- $\alpha$  gene. It was not necessary to separate the two heteroduplex forms since the deoxyoligonucleotides were complementary to the “+” strand only.

After annealing, 300  $\mu$ l of 0.3 NaAc, pH 7.0 and 20  $\mu$ g of carrier tRNA were added and the heteroduplex molecules were precipitated two times with ethanol. After the second centrifugation, the pellet was rinsed with cold ethanol and dried under vacuum. The heteroduplex forms were purified by electrophoresis through a 0.7% agarose gel. The heteroduplex forms migrated with nicked parental plasmid, but slower than both linearized forms.

To recover the heteroduplex forms, it was necessary to use electroelution. The gel strip containing the heteroduplex band was placed in a dialysis bag with 1 ml of TAE, pH 7.8, running buffer with ethidium bromide (50  $\mu$ g/ml). The heteroduplex molecules were visualized under long wavelength UV as they migrated out of the gel strip into the buffer. After electrophoresis, the elution buffer was recovered and combined with four 1 ml rinses of the dialysis bag with a solution consisting of 10 mM Tris · HCl, pH 7.4, 1 M EDTA, and 300 mM NaCl. 2-Butanol was used to reduce the volume of 0.5 ml. Ten micrograms of carrier tRNA was added and the heteroduplexes were extracted once with 500  $\mu$ l of phenol/chloroform/isoamyl alcohol (50:50:1), precipitated two times with ethanol, dried, and resuspended in 3  $\mu$ l of H<sub>2</sub>O. About 50 ng of heteroduplexes (as measured by visual comparison with standardized plasmid DNA on an agarose gel) was recovered from the gel. Electroelution from agarose was necessary due to the strong affinity between the single-stranded region of the heteroduplex and agarose. It was also important not to filter the eluted heteroduplex through nitrocellulose because single-stranded DNA has high affinity for the filter.

Introduction of Deletions in IFN- $\alpha$  with Synthetic DNA

Deletions in plasmid DNA were used to produce truncated IFN- $\alpha$  proteins which were terminated after Cys 138, Val 143, or Arg 149 (Fig. 1a). The three deoxyoligonucleotides shown in Table II each directed a loop-out of sequences in the “+” strand to position the TGA stop codon immediately downstream of the sequences encoding Cys 138, Val 143, or Arg 149. By designing the synthetic DNAs to complement 12 nucleotides in the “+” strand on the 5' side of the splice point and 12 nucleotides on the 3' side, the intervening sequences not complementary to the deoxyoligonucleotide will loop-out as illustrated in Fig. 4.

Parental	Lys	Tyr	Ser	Pro	Cys	Ala	Trp	Glu	Val	Val	Arg	Ala	Glu	Ile	Met	Arg	Ser		
	<div style="display: flex; justify-content: space-between; width: 100%;"> <span>138</span> <span>143</span> <span>149</span> <span>166</span> </div>																		
"+" 5' . . .	AAA	TAC	AGC	CCT	TGT	GCC	TGG	GAG	GTT	GTC	AGA	GCA	GAA	ATC	ATG	AGA	TCT	. . .	GAA TGA AAA CTG GTT
	<div style="display: flex; justify-content: space-between; width: 100%;"> <span>Cys</span> <span>END</span> </div>																		
Δ138	3' ATG TCG GGA ACA ACT TTT GAC CAA																		
	<div style="display: flex; justify-content: space-between; width: 100%;"> <span>Val</span> <span>END</span> </div>																		
Δ143	3' ACC CTC CAA CAG ACT TTT GAC CAA																		
	<div style="display: flex; justify-content: space-between; width: 100%;"> <span>Arg</span> <span>END</span> </div>																		
Δ149	3' CTT TAG TAC TCT ACT TTT GAC CAA																		

Δ138 directs a loop-out of 81 bases; Δ143 directs a loop-out of 66 bases; Δ149 directs a loop-out of 48 bases.

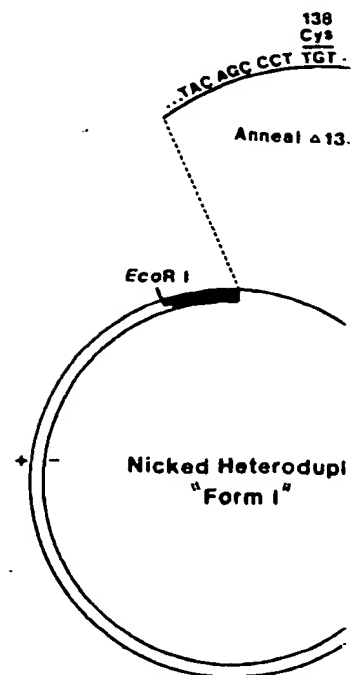


FIG. 4. A synthetic DNA ( $\Delta 138$ ) terminal coding sequences. In this case, the IFN- $\alpha$ A sequences not complementary to the IFN- $\alpha$ B results from the replication of the "

To effect mutagenesis, the "+" strand in the Form I heteroduplex was annealed to a 50-fold molar excess of phosphorylated oligonucleotide combined with the purified heteroduplex. The mixture was heated to 7.4. The mixture was heated and cooling prevented the complete heteroduplex forms from self-annealing. After several minutes at 0°, the volume was adjusted to 1 mM Tris · HCl, pH 7.4, 50 mM NaCl, 5 mM ATP, 400 μM of each of the dNTPs, and 10 units of Klenow fragment of *E. coli* DNA Polymerase I and 10 units of DNA Ligase. Filling-in of the strand was performed at 15° for 1 hour.

<sup>17</sup> R. B. Wallace, M. Schold, M. J. Jo  
9, 3647 (1981).

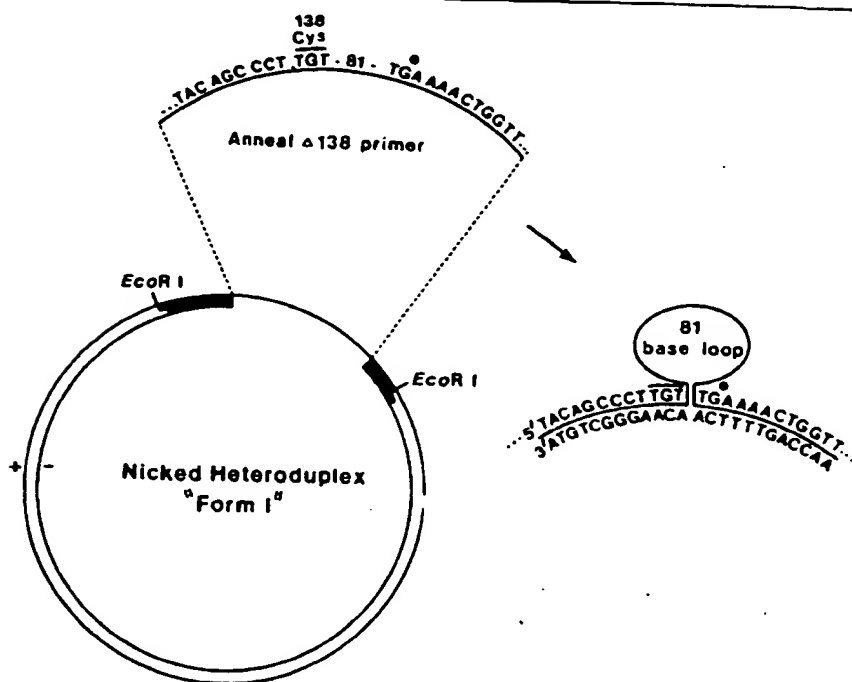


FIG. 4. A synthetic DNA ( $\Delta 138$ ) directs the precise splicing of the IFN- $\alpha$ A carboxy-terminal coding sequences. In this example, a deletion of 81 bp resulted from a loop-out of IFN- $\alpha$ A sequences not complementary to the deoxyoligonucleotide. The mutant plasmid results from the replication of the "+" strand.

To effect mutagenesis, the deoxyoligonucleotide was annealed to the "+" strand in the Form I heteroduplex as has been described.<sup>17</sup> Briefly, a 50-fold molar excess of phosphorylated deoxyoligonucleotide was combined with the purified heteroduplex in a volume of 5  $\mu$ l of 0.1  $\times$  TE, pH 7.4. The mixture was heated at 68° for 3 min and quenched at 0°. The rapid cooling prevented the complementary single-stranded regions of both heteroduplex forms from self-annealing to displace the primer. After several minutes at 0°, the volume was adjusted to 20  $\mu$ l by the addition of 10 mM Tris  $\cdot$  HCl, pH 7.4, 50 mM NaCl, 100 mM MgCl<sub>2</sub>, 1 M DTT, 400  $\mu$ M ATP, 400  $\mu$ M of each of the four deoxynucleotide triphosphates, 3 units of Klenow fragment of *E. coli* DNA Polymerase I, and 120 units of T<sub>4</sub> DNA Ligase. Filling-in of the single-stranded region and subsequent ligation was performed at 15° for 6 hr. The reaction volume was then adjusted

<sup>17</sup> R. B. Wallace, M. Schold, M. J. Johnson, P. Dembek, and K. Itakura, *Nucleic Acids Res.* 9, 3647 (1981).

Val END

 $\Delta 143$  3' ACC CTC CAA CAG ACT TTT GAC CAA

ARG END  
 $\Delta 149$  3' CTT TAG TAC TCT ACT TTT GAC CAA

\*  $\Delta 138$  directs a loop-out of 81 bases;  $\Delta 143$  directs a loop-out of 66 bases;  $\Delta 149$  directs a loop-out of 48 bases.

to 250  $\mu$ l by the addition of 0.3 M sodium acetate, pH 7.0, and extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). The DNA was precipitated twice with ethanol, dried, and resuspended in 5  $\mu$ l of H<sub>2</sub>O for transformation of *E. coli* MC1061.<sup>18</sup> The efficiency of obtaining a mutation was about 5–15% of the colonies screened. Each positive colony detected by mini-prep plasmid preparation had two plasmids present, one plasmid with the deletion and the parental plasmid. Thus, it was necessary to retransform with plasmid DNA to select those colonies which contain the plasmid with the deletion. Similarly, analogs of IFN- $\alpha$ A prematurely terminated at Val 143 and Arg 149 were prepared.

#### Analysis of Prematurely Terminated IFN- $\alpha$ A Proteins

The results obtained above for the Cys 1 and Cys 98 substitutions were indirect evidence for the importance of Cys 29/Cys 138 intramolecular disulfide bond formation since fully reduced IFN- $\alpha$ A retains only 5% of its activity in the MDBK cell assay.<sup>19</sup> Therefore, it was interesting to determine if amino acids distal to Cys 138 were necessary for biological activity.

*E. coli* cells which contained plasmids with the specific deletions were induced at 42° and extracts were prepared by 7 M guanidine hydrochloride lysis as described.<sup>2</sup> Each extract demonstrated no biological activity in the MDBK cell assay. To determine whether these analogs were inactive as the direct result of the carboxy-terminal deletions or because of *in vivo* instability, plasmids encoding the Cys 138, Val 143, Arg 149, and parental IFN- $\alpha$ A proteins were used in a cell-free, prokaryotic DNA-dependent transcription/translation system.<sup>20</sup> The IFN- $\alpha$ A analogs were synthesized to a level at least equal to that of parental IFN- $\alpha$ A, but were biologically inactive. This result suggests that amino acids distal to the Arg residue at position 149 in the primary structure are required for maximal antiviral activity of IFN- $\alpha$ A.

#### Concluding Comments

This chapter illustrates two approaches for mutagenesis. The approach used for generating the Cys 1 and Cys 98 substitutions requires extensive synthesis of deoxyoligonucleotide duplexes. The heteroduplex

<sup>18</sup> M. J. Casadaban and S. N. Cohen, *J. Mol. Biol.* **138**, 179 (1980).

<sup>19</sup> J. Langer and S. Pestka, this volume [34].

<sup>20</sup> H.-F. Kung, C. Spears, and H. Weissbach, *J. Biol. Chem.* **250**, 1556 (1975).

method requires only a single synthesis can be performed between restriction sites 12% of the plasmid length. The formation of the heteroduplex

Deletions made in the plasmid provided evidence that amino acids are required for full IFN- $\alpha$ A activity with these preparations (data not shown). Cys 138 and Arg<sup>149</sup> were assayed, the efficiency of a molecule to terminate cells. Zoon *et al.*<sup>22</sup> reported that thermolysin digestion contained exhibited about 1/100th of the activity of the cells. Recently, Nisbet *et al.* in IFN- $\alpha$ 1 that showed significant activity. It was reported by Levy *et al.* that interferon closely corresponds to position 155, 10 amino acids from the fully active interferon molecule. The carboxy-terminal 10–13 amino acids of IFN- $\alpha$ A activity.<sup>25,26</sup> This information presented here, suggests that the carboxy-terminal region (i.e., amino acids required for maximal antiviral

#### Acknowledgments

We would like to thank Drs. F. Pennina Langer-Safer for helpful discussions of *E. coli* cells. Many thanks to the manuscript.

<sup>21</sup> Y. Morinaga, T. Franceschini, S. (1984).

<sup>22</sup> S. A. Ackerman, D. Z. Nedden, *Natl. Acad. Sci. U.S.A.* **81**, 1045 (1984).

<sup>23</sup> I. T. Nisbet, M. W. Beilharz, P. J. *Int.* **11**, 301 (1985).

<sup>24</sup> W. P. Levy, M. Rubinstein, J. Shiv, Gerber, S. Stein, and S. Pestka, *P.*

<sup>25</sup> A. E. Franke, H. M. Shepard, C. Lawn, *DNA* **1**, 223 (1982).

<sup>26</sup> N. Chang, H.-F. Kung, and S. Pestka

acetate, pH 7.0, and extracted with chloroform/isoamyl alcohol twice with ethanol, dried, and ligation of *E. coli* MC1061.<sup>18</sup> The about 5–15% of the colonies by mini-prep plasmid preparation with the deletion and the to retransform with plasmid the plasmid with the deletion. terminated at Val 143 and Arg

### IFN- $\alpha$ Proteins

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138, 179 (1980).

*J. Biol. Chem.* 250, 1556 (1975).

method requires only a single synthetic deoxyoligonucleotide and mutagenesis can be performed directly on the expression plasmid. The distance between restriction sites used for making the gap is limited to about 12% of the plasmid length. While this work was in progress, an improvement of the heteroduplex method was reported.<sup>21</sup>

Deletions made in the IFN- $\alpha$ A gene carboxy-terminal coding region provided evidence that amino acid residues distal to Arg<sup>149</sup> are required for full IFN- $\alpha$ A activity when measured on MDBK cells. When purified preparations (data not shown) of IFN- $\alpha$ A molecules terminated at Cys<sup>138</sup> and Arg<sup>149</sup> were assayed, they showed approximately 1/100th of the activity of a molecule terminated at Phe<sup>151</sup> and full length IFN- $\alpha$ A on MDBK cells. Zoon *et al.*<sup>22</sup> reported that a fragment of IFN- $\alpha$ A produced by thermolysin digestion containing the amino-terminal 110 amino acids also exhibited about 1/100th of the activity of the intact molecule on MDBK cells. Recently, Nisbet *et al.*<sup>23</sup> made a glycine substitution for tyrosine 136 in IFN- $\alpha$ 1 that showed significant loss of antiviral activity on bovine cells. It was reported by Levy *et al.*<sup>24,25</sup> that two natural species of leukocyte interferon closely corresponding to IFN- $\alpha$ A in sequence terminated at position 155, 10 amino acids shorter than IFN- $\alpha$ A. These species were fully active interferon molecules. In addition, it has been reported that the carboxy-terminal 10–13 amino acids can be eliminated without loss of IFN- $\alpha$ A activity.<sup>25,26</sup> This information, taken together with the results presented here, suggests that structural features contributed by the carboxy-terminal region (i.e., amino acids 151–154) of IFN- $\alpha$ A may be required for maximal antiviral activity on MDBK cells.

### Acknowledgments

We would like to thank Drs. Richard Kramer, Robert Crowl, Kenneth Collier, and Pennina Langer-Safer for helpful discussions and Mr. Dale Mueller for large-scale fermentations of *E. coli* cells. Many thanks to Ms. Sharon Smith for excellence in preparing the manuscript.

<sup>21</sup> Y. Morinaga, T. Franceschini, S. Inouye, and M. Inouye, *BiolTechnology* July, 636 (1984).

<sup>22</sup> S. A. Ackerman, D. Z. Nedden, M. Heintzelman, M. Hunkapiller, and K. Zoon, *Proc. Natl. Acad. Sci. U.S.A.* 81, 1045 (1984).

<sup>23</sup> I. T. Nisbet, M. W. Beilharz, P. J. Hertzog, M. J. Tynus, and A. W. Linnane, *Biochem. Int.* 11, 301 (1985).

<sup>24</sup> W. P. Levy, M. Rubinstein, J. Shively, U. Del Valle, C.-Y. Lai, J. Moschera, L. Brink, L. Gerber, S. Stein, and S. Pestka, *Proc. Natl. Acad. Sci. U.S.A.* 78, 6186 (1981).

<sup>25</sup> A. E. Franke, H. M. Shepard, C. M. Houck, D. W. Leung, D. V. Goeddel, and R. M. Lawn, *DNA* 1, 223 (1982).

<sup>26</sup> N. Chang, H.-F. Kung, and S. Pestka, *Arch. Biochem. Biophys.* 221, 585 (1983).

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